

Growth enhancement of black pepper (*Piper nigrum*. L) by a newly isolated *Bacillus tequilensis* NII-0943

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Abstract

A Gram positive, rod shaped potential strain selected from the pool of a bacterial isolates obtained from the Western *ghat* forest on the basis of zone of P-solubilization activity. Identification based on 16S rRNA gene sequence reveals that, the strain is of *Bacillus* species, which shares highest sequence similarity to *Bacillus tequilensis* NRRL B-41771^T (99.5%). Strain NII-0943 was able to produce good amount of indole acetic acid (IAA) and positive for siderophore production. In addition to IAA and siderophore attributes, strain NII-0943 also possessed the characteristics like tri-calcium phosphate solubilization and growth in nitrogen free medium. Seed inoculation with the strain NII-0943, resulted in significantly higher root initiation in black pepper cuttings grown under pots. The contents of nitrogen and phosphorus in soil, and plant were also enhanced significantly in treatments inoculated with these bacterial inocula. Hence, based this evidence it is proposed that, strain NII-0943 could be deployed as a plant growth promoting inoculant to attain the desired results of bacterization.

Keywords: *Bacillus tequilensis*, Plant growth activity, Black pepper, 16S rRNA gene

Abbreviations: PGPB-Plant growth promoting bacteria, NA-nutrient agar, NBRIP-National Botanical Research Institute's phosphate growth medium, IAA-Indole acetic acid, HCN-Hydrogen cyanide, CAS-Chrome Azurol-S assay

Introduction

Black pepper is native to South India and is extensively cultivated there and elsewhere in tropical regions. Black pepper (*Piper nigrum*) is a flowering vine in the family *Piperaceae*, cultivated for its fruit, which is usually dried and used as a spice and seasoning. Black Pepper is a plant of humid tropics requiring adequate rainfall and humidity. The hot and humid climate of sub-mountainous tracts of Western *ghat* in India is an ideal place for its cultivation. It grows successfully between 20° North and South latitude and up to 1500 meters above the sea level (Sarma 2003). The crop tolerates temperature between 10° and 40°C. Nitrogen and phosphorus are essential nutrients for plant growth and development. Intensive agriculture entails the risk of excessive fertilization (Diby and Sarma 2006).

Plant growth-promoting bacteria (PGPB) are able to exert a beneficial effect upon plant growth (Jeffries et al. 2003). PGPB can promote plant growth directly or indirectly, via biocontrol of host plant diseases, production of phytohormones, or improvement of plant nutritional status (Glick 1995). Rhizobia are perhaps the best known beneficial plant associated bacteria because of the importance of the nitrogen fixation that occurs during the Rhizobium–legume symbiosis. The co-inoculation of other PGPB with rhizobia is becoming a practical method in the development of sustainable agriculture, because of yield increases seen compared with inoculation with rhizobia alone (Jeffries et al. 2003; George et al. 2005; Diby and Sarma 2006). PGPB that have been tested as co-inoculants with rhizobia include strains of the following well-known bacteria: *Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Serratia* and *Streptomyces* (Baudoin et al. 2009, Biari et al. 2008, Ahmad et al. 2008). In the present study we reporting on a potential isolate of bacillus sp isolated from Western *ghat* dense forest with their effective in-vivo plant growth promoting activity in black pepper.

Materials and Methods

Isolation and characterization of bacteria

The soil used for bacterial isolation was collected from a root-free soil of Silent valley in Western *ghat* [GPS coordinates for the sample site are 74° 52'E, 8°18'N] located in the Nilgiri Hills, Palakkad district, Kerala, in South India. The rich forests of the Western *ghat* harbor a large portion of India's biological diversity and include most of the endemic species. The processed soil sample was serially diluted, spread plated on nutrient agar (NA) (g⁻¹, beef extract-3, peptone-5 and agar-20, pH 7.0) and incubated at 28°C for 48 h. A total of 520 different colonies were isolated on nutrient agar and were purified with

repeated culturing and maintained in 20% glycerol at -80°C . Potential isolates were screened and selected on the basis of halo zone produced in Pikovskaya agar (Pikovskaya 1948). To investigate its morphological, physiological and biochemical characteristics, strain was routinely cultivated at 28°C on NA agar and as glycerol suspensions (20 %, v/v) at -80°C . Gram staining was performed using a bioMérieux Gram-stain kit according to the manufacturer's instructions. Cell morphology and motility were studied using phase-contrast microscopy and scanning electron microscopy (JEOL apparatus Philips SEM 515). The physiological characteristics of strain NII-0922^T were examined by growing the isolate on NA medium and biochemical tests using Hi-25 Kit (HiMedia, Mumbai). The temperature range for growth was checked on nutrient broth inoculated with bacterial inocula and incubated at 5 to 50°C at an interval of 5°C . Results were recorded after every 4h at 600nm. The ability of the isolate to grow in different salt concentrations was carried out by inoculating bacterial culture on NA plates supplemented with 0-25% (w/v, with 1 unit interval) NaCl and the plates were incubated at $28 \pm 1^{\circ}\text{C}$ for three days. The ability of the isolates to grow in alkaline or acid media was tested in nutrient broth in which the pH was adjusted from 4.0 to 12.0 (at a pH 1.0 interval) using the different buffer system: pH 4.0–5.0: 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0: 0.1 M KH_2PO_4 /0.1 M NaOH; pH 9.0–12.0: 0.1 M NaHCO_3 /0.1 M Na_2CO_3 and incubated at $28 \pm 1^{\circ}\text{C}$ for three days. The ability to use different carbon sources was tested using the Biolog GN Microplate method (Biolog, Hayward, CA). Strain NII-0943 was then screened for traits that might be associated with ability to functions as PGPB, each test performed in triplicate. The identification of the isolate upto species level was revealed on the basis of Biolog carbon source utilization, and further validated by the 16S rRNA gene sequencing.

DNA Extraction and 16S rRNA Sequencing

DNA was extracted using the method described by Marmur (1962). 16S rRNA gene sequencing performed using an ABI PRISM BigDye Terminator cycle sequencing kit (as recommended by the manufacturer). The universal primer 27F and 1492R was used for the partial sequencing of the 16S rRNA gene. Sequence was deposited in GenBank under FJ897473 accession. Sequence analysis was done at the RDP-II (Ribosomal Database Project, Michigan State University, MI, USA) using Seqmatch version 3 (Cole et al. 2005). Similarity scores were obtained by the similarity rank analysis function at RDP data version 9.50. Nucleotide sequences were aligned using the Clustal X 1.81 algorithm (Thompson et al. 1997). Phylogenetic and molecular evolutionary analyses were conducted using mega version 4.0 (Kumar *et al.* 2004). The phylogenetic tree were constructed by the neighbor- joining

method (Saitou and Nei 1987) using the distance matrix from the alignment. Distances were calculated using the Kimura method (1980).

Amplification of the 16S rRNA gene fragment was carried out using universal primers (27F and 1492R). 16S rRNA gene sequences of the isolates were compared with 16S rRNA sequences available by the BLAST search in the NCBI, GenBank database (<http://www.ncbi.nlm.nih.gov>) (Altschul et al. 1990). Multiple sequence alignments were performed using CLUSTALW (Thompson et al. 1997). The method of Jukes and Cantor (1969) was used to calculate evolutionary distances. Phylogenetic dendrogram was constructed by the neighbour-joining method (Saitou and Nei 1987) and tree topologies were evaluated by performing bootstrap analysis of 1,000 datasets using MEGA 3.1 (Kumar et al. 2004). Distances were calculated using the Kimura method (1980). The sequences were submitted to NCBI GenBank.

Quantitative estimation of P-solubilization, Indole acetic acid, siderophore and HCN

The quantitative estimation of P-solubilization carried out as per standard methodology (Nautiyal 1999), by inoculating 1 mL of bacterial suspension (3×10^7 cells mL⁻¹) in 50 ml of National Botanical Research Institute's phosphate growth medium (NBRIP broth) in Erlenmeyer flasks (150mL), and incubating the flasks for 15 days. Every three days of the incubation period the cell suspension was centrifuged at 10,000 rev min⁻¹ for 10 min. and the P content in the supernatant was spectrophotometrically estimated by the ascorbic acid method (Murphy and Riley 1962). All the studies were repeated on three independent dates to confirm the results. In order to observe the effect of cultural conditions for insoluble phosphate solubilization, the bacterial cells were cultured at four different temperature (5, 20, 30 and 40°C) and pH (pH 3 to 11 range with a 1 unit interval).

Indole acetic acid (IAA) quantification was carried with liquid medium supplement with 1g tryptophan and was inoculated by bacterial culture adjusted to optical density 0.5 measured at 600 nm by spectrophotometer and were incubated at 30°C for 24–48 h. After incubation cells were removed by centrifugation (5,000 rev/min for 15 min). Auxin was detected in 1 mL of supernatant using Salkowski reagent (Gordon and Weber 1951; Sarwar et al. 1992). A standard curve was drawn to determine auxin production by isolates. The presence of IAA was further confirmed by HPLC. Filter-sterilized supernatant was analysed by using a Shimadzu HPLC equipped with a Hypersil-Keyston ODS column (5 mm; 4.6 · 250 mm). The mobile phase was methanol/water/acetic acid (36:64:1) at a flow rate of 1

mL/min. Eluates were detected at 220 nm and IAA was quantified by integrating the areas under the peaks. Authentic IAA (Sigma) was used as standard.

For qualitative estimation for siderophore and hydrogen cyanide (HCN) production, isolates were initially screened qualitatively for production of cyanide by using picrate/ Na_2CO_3 saturated filter paper fixed to the underside of Petri dish lids (Bakker and Schipper 1987), which were sealed with parafilm before incubation at 28°C. Color change of the filter paper from yellow to light brown, brown, or reddish brown was recorded at 4, 24 and 48 h as an indication of weak, moderate, or strong cyanogenic potential, respectively. Reactions from inoculated plates were visually compared with corresponding control plates containing no culture. Siderophore production was detected by the standard Chrome Azurol-S (CAS) assay (Pankaj et al. 2007) in 110 mm Petri dishes, and the diameter of the clearing zone was measured. The composition of CAS blue solution for this assay was prepared according to Schwyn and Neilands (1987). HCN production was inferred by the qualitative method of Bakker and Schipper (1987). The change in the color of the filter paper previously dipped in 2% sodium carbonate prepared in 0.05% picric acid, from yellow to dark brown was rated visually depending on the intensity of the colour change. Pure isolates were stabbed on CAS agar plates using sterile toothpicks and incubated at 28°C for 2 weeks in the dark. The colonies with orange zones were considered as siderophore-producing strains. Assay in solid media were carried out in triplicate. The control plates of CAS-agar (uninoculated) were incubated under the same conditions as described above and no color change in the CAS-blue agar was observed, after incubation periods of 1–14 days.

Black pepper bioassays

For the bioassays, stem cuttings (15 cm in height) of the black pepper cultivar ‘Panniyur-6’ were obtained from runner shoots of plants from a healthy black pepper orchard. To study the plant growth-promoting activities of the selected bacterial isolates, two types of natural soil were collected from a garden field in NIIST institute campus. Black pepper was not grown in this soil before. One soil is acidic [pH 4.7, organic carbon 0.15%, P-5.30(kg/ha), K- 54.0(kg/ha)] other one is alkaline [pH 7.2, organic carbon 2.6%, P-537.6 (kg/h), K-448.0(kg/h)] as analysed in soil testing laboratory of Agriculture College Vellayani, Trivandrum. Both soil samples were mixed with sand in the ratio 1:1 (v / v) and sterilized for 30 minutes at 121°C for three consecutive days and then transferred to plastic pots. For the bioassays, the disease-free cuttings (15 cm height) were surface-sterilized with ethanol (70%) for 5 min followed by several washings with ample amounts of sterile water. Excess water adhering to the cuttings

was removed using sterile facial tissues. Fresh cuts were made at the ends of the cuttings to yield sterile end tissues. The lower half of the stem cuttings, including the first node, was dipped in water (control) or bacterial suspensions (10^8 cfu mL⁻¹) for 30 min prior to transplanting to the pots. The bioassays were conducted in the greenhouse with temperature and humidity control ($28 \pm 2^\circ\text{C}$ and 60% humidity). The population dynamics of the bacterial isolates on the roots of black pepper seedlings was assessed after 60 days of plant growth in the greenhouse. Population densities of the introduced bacterial strains were assessed by dilution plating of rhizosphere suspensions on nutrient agar medium. The height of the new black pepper shoots was scored after 60 days of plant growth. Additionally, the number of roots per cutting and the length of the roots were determined at 60 days. From the samples of rhizosphere soil, sub-samples were aseptically separated from roots, and the soil chemical properties were determined. Soil pH was measured in 1:2.5 soil/deionized water. Inorganic phosphorus (labile forms of P) was extracted by the bicarbonate method and analyzed by the molybdate-blue method (Murphy and Riley 1962). Total available P was determined after extraction by the bicarbonate method and digestion with strong acids (perchloric/nitric; (Cross and Schlesinger 1995). Exchangeable cations (K, Ca, Mg, and Na) were extracted with 1 M CH₃COONH₄ at pH 7.0 and analyzed by flame atomic absorption spectrophotometry (FAAS). Exchangeable aluminum was extracted with 1 M KCl and analyzed by FAAS.

Statistical analysis

Data were statistically analyzed by analysis of variance using the general linear model developed by the SAS Institute (version 9.1; Cary, NC), and means were compared using the least significant difference (LSD) method; $P \leq 0.05$ was considered significant.

Results

Isolation and Characterization of potential bacteria

A potential bacterial strain producing about an 8.0cm zone of phosphate solubilization after 48h of incubation on Pikovskaya agar was subjected for further assessed for phenotypic and physiological characteristics. The bacterial colonies were circular, smooth, convex, and entire. The strain was Gram-positive and able to grow over a wide range of temperature 5-45°C, with an optimum at $28 \pm 2^\circ\text{C}$ and over a pH range of 4-11, with optimum 7.0 ± 0.5 and could tolerate 0-15% of NaCl (w/v). Phenotypic and biochemical characterizations were done using Biolog based carbon source utilization and Hi25 Kit

(HiMedia, Mumbai), strain utilizes arabinose, sucrose, sorbose, rhamnose, xylose, maltose, glucose, fructose, alanine, tyrosine, glutamate and tryptophan. The strain does not utilize erythritol, glycogen, leucine, glycine, histidine and cellobiose. The strain is positive for indole production, oxidase reduction, H₂S production, Catalase activity, tween 80, nitrate reduction, casein and starch hydrolysis. But negative for gelatin liquefaction, citrate utilization, DNase activity, cellulose degradation, asparaginase, lipase, esculin and urease. No growth observed at anaerobic conditions. Molecular analysis based on 16S rRNA gene sequencing reveals that isolate NII-0943 showed maximum similarity to *Bacillus tequilensis* NRRL B-41771(99.5%), available in the public domain. Based on 16S rRNA gene sequences a phylogenetic tree was constructed that positions the potential isolates to their respective group (**Figure.1**)

Plant growth promoting attributes

The isolate was found to solubilize phosphate significantly at 28±2°C. Although the zone of solubilization around the bacterial colony on Pikovskaya agar after 72h of incubation varies from 5°C to 40°C. Quantitative estimation of phosphate solubilization, estimated after incubation for 15 days (at 3, 5, 7, 10, 15 days intervals), maximum solubilization was observed at 30°C (**Table.1**)

At this temperature, maximum solubilization of 28.3 µg mL⁻¹ was recorded after 10th day of incubation with NII-0943, after which the solubilization continued to decline with increased incubation. The pH of the broth was found to decline and it is observed that, lowering of pH coincided with increase in the efficiency of phosphate-solubilizing activity. Maximum decline in pH was found to be < 5.0 from initial pH 7.0 To determine the optimum temperature for phosphate solubilization, shake flask cultures were carried out at four different temperatures (5, 20, 30 and 40°C). And maximum IAA production was 127.5µg mL⁻¹ in tryptophan amended media at 30°C after 48h of incubation. The strain was able to grow at all the temperatures tested, and grew especially well at 30°C. The survival of the isolate under different temperatures was of significance. Although more research is needed to evaluate its efficiency under temperature stress conditions, it was well documented that the relative effectiveness of plant inoculation was higher under extreme conditions of soil temperature in different experiments. The influence of initial pH on the insoluble phosphate solubilization was investigated in the range of pH 3.0 to 11.0. The bacterial growth and phosphate solubilization were varied in all pH units tested. However, pH 3.0 and above 9.0 resulted less bacterial growth. P-solubilization in pH 3.0 is very high, this result strongly supports the observations as pH decreases solubilization of phosphate increases. These results

indicated that isolates of Western *ghat* are acid- and alkali-tolerant bacterium which can be applied to both kinds of soils.

Plant yield parameters

The strain NII-0943 exerted a considerable influence on growth of black pepper cuttings and recorded with higher root numbers, shoot length and root length compared to untreated ones (control), black pepper cuttings showed 77% and 112.5% of more root and shoot length respectively and exhibited improved uptake of three major nutrients Seed bacterization resulted in greater enhancement of the root growth, as compared with the shoot growth. **(Figure 2 & 3)**

Discussion

The production of plant growth regulators (PGRs) has been suggested to be one of the mechanisms by which plant-growth-promoting microorganisms stimulate plant growth. Diverse soil microorganisms including bacteria (Arshad and Frankenberger 1991; Khalid et al. 2004), filamentous fungi (Kaldorf and Ludwig-Muller 2000; Le Floch et al. 2003) and yeasts (El-Tarabily 2004) are capable of producing physiologically active quantities of auxins and which have pronounced effects on plant growth and development. L-Tryptophan (L-TRP) is considered as a physiological precursor of auxin biosynthesis in both higher plants and microorganisms (Park et al. 2005; Tsavkelova et al. 2005). Exogenous application of L-TRP substantially increased auxin production in vitro by various bacteria (Khalid et al. 2004) and filamentous fungi (Frankenberger and Poth 1987). Biosynthesis of IAA in bacteria is able to make physiologically active IAA that may have pronounced effects on plant growth and development (Costacurta and Vanderleyden 1995). About 80% of bacteria isolated from plant rhizospheres are able to produce indole-3-acetic acid (Costacurta and Vanderleyden 1995). With the analysis of bacterial species, the role of bacterial IAA in plant–microorganism interactions appears to be diverse. Previously, bacterial auxin production was mainly associated with pathogenesis, specifically with bacterial gall formation. However, it became apparent that many of the phytopathogenic (not only gall-inducing) as well as plant growth-promoting bacteria have the ability to synthesize IAA (Berleth and Sachs 2001). The broad distribution of IAA biosynthesis genes across bacteria, the existence of different metabolic pathways and the diversity in outcomes on the plant side when IAA-synthesizing bacteria interact with plants, evoke the question as to why bacteria produce IAA. Except for a few cases, the link between IAA synthesis and plant, phenotype has not been demonstrated or at least remains ambiguous. For various PGPR, it has

been demonstrated that enhanced root proliferation is related to bacterial IAA biosynthesis (Tsavkelova et al. 2007). Studies with *Azospirillum* mutants altered in IAA production support the view that increased rooting is caused by *Azospirillum* IAA synthesis (Dobbelaere et al. 1999). This increased rooting enhances plant mineral uptake and root exudation, which in turn stimulates bacterial colonization and thus amplifies the inoculation effect (Dobbelaere et al. 1999; Lambrecht et al. 2000). However, in several studies it was shown that IAA biosynthesis alone cannot account for the overall plant growth-promoting effect observed. Therefore, the 'additive hypothesis' was suggested to explain growth and yield promotion with PGPR, postulating that growth promotion is the result of multiple mechanisms (phytohormone biosynthesis, N-fixation, P-solubilization) working together (Bashan and Holguin 1997). Similarly, the capacity of *Pseudomonas putida* GR12-2 to stimulate root elongation was shown to be related to its production of IAA but production of IAA alone does not account for growth promotion, as evidenced by a study with IAA overproducing mutants (Xie et al. 1996). In their study transposon mutagenesis was used to isolate seven mutants that overproduced IAA in comparison with the wild-type. Interestingly, six of the seven mutants, including one which produced IAA at three times the rate of GR12-2, elicited root elongation at a level statistically equivalent to GR12-2. Large amounts of IAA produced by bacteria together with endogenously produced plant IAA activate ACC synthase, leading to production of ACC, a precursor for ethylene. Ethylene is an inhibitor of root growth especially reducing the primary root length (Glick et al. 1998; Glick 2005). Idris et al. (2007) demonstrated the relationship of plant growth efficiency with IAA synthesizing ability of *Bacillus amyloliquefaciens*.

In present study we demonstrated that a newly isolated *Bacillus* sp NII-0943 shows a potential plant growth promoting activity in black pepper both in acidic as well as in alkaline soil conditions. The strain produces 127.5µg/mL of IAA which results in proliferation of root elongation in black pepper. The strain also shows the uptake of three major macro nutrients and resulted in P-solubilization (28.35µg/ml). In the end, it can be concluded that the bacterial strain isolated from the Western *ghat* forest soil is enhanced in IAA content and this trait can be used for the selection of effective PGPR. Pot experiments demonstrated that the strain NII-0943 has the potential to increase the growth as well as the yield of black pepper (*Piper nigrum*) and offers a good opportunity to be used as biofertilizers to increase the growth and yield of agronomically important crops in different soil types.

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Legends for Tables and Figures

Table. 1. Plant growth promoting attributes of *Bacillus* sp NII-0943 at different incubation temperature. (Values are mean of three independent observations, +, moderate; ++ strong activity)

Figure. 1. Neighbour-joining phylogenetic dendrogram based on 16S rRNA gene sequence. Numbers at the branch nodes are bootstrap values (per 1000 trials). Only values greater than 50 are indicated. The bar represents 0.002% base substitution.

Figure. 2. Black pepper cutting showing increased proliferation of roots and leaves when treated with *Bacillus* sp NII-0943 over untreated cuttings after 60 days of cultivation in pots. [A-control (untreated-no roots observed); B, C, D-Treated with NII-0943 (triplicates, n=3)]

Figure. 3. A. Increased length of root, shoot and number of roots when treated with NII-0943 compared with untreated. B. Nutrient uptake parameters of black pepper when treated with strain NII-0943. [Values are means of three replicates of three independent experiments (n=3) ± standard deviation Significantly different from the control at $P \leq 0.05$]

Table.1

Incubation temperature (°C)	P-solubilization Ca ₃ (PO ₄) ₂ μgmL ⁻¹ day ⁻¹	IAA	HCN Production	Siderophore
5	607±1.2	12.3±0.9	–	+
20	10.75± 0.3	89.6±1.0	++	++
30	28.30±0.8	127.5±1.2	++	++
40	5.61±0.5	46.0±1.0	++	+

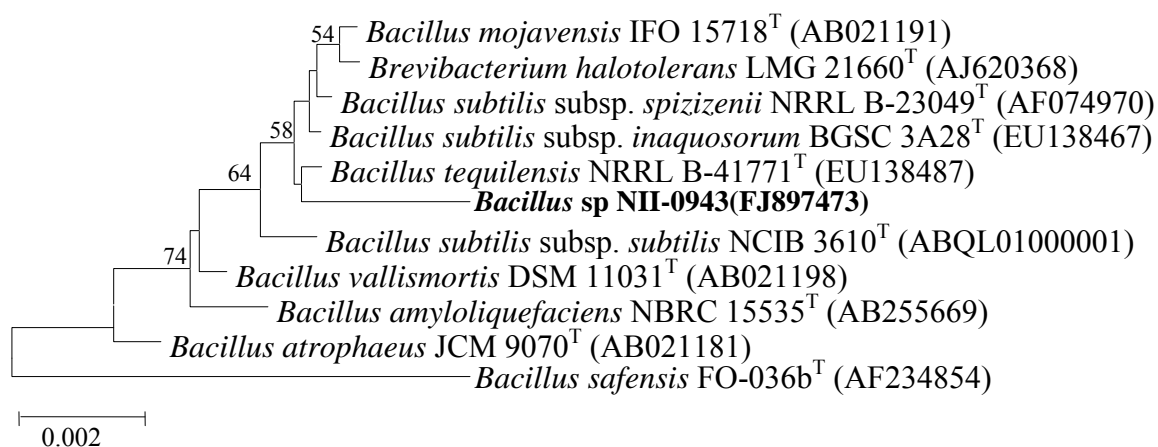
Figure.1.

Figure.2.

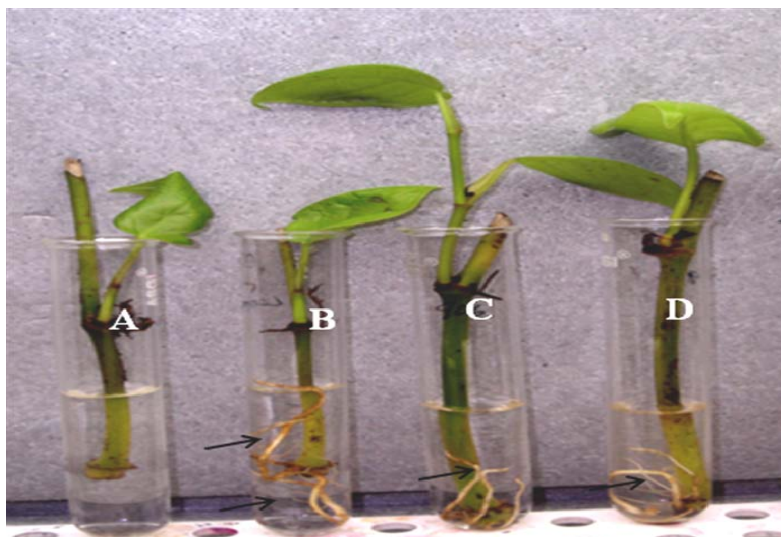


Fig.3.

